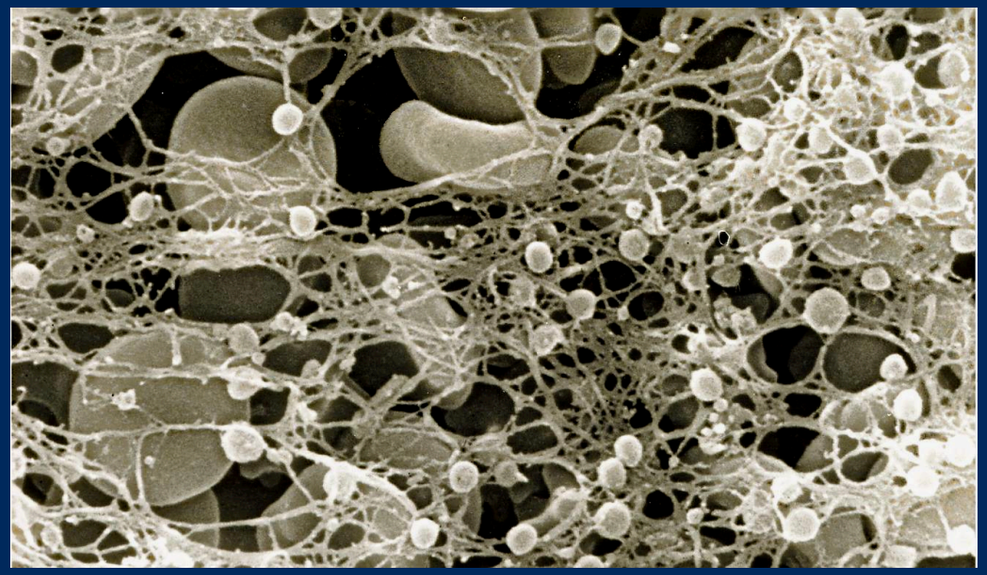
Thesis for doctoral degree (Ph.D.)
2012

Characterization of extracellular and surface bound adherence proteins of *Staphylococcus aureus*



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Characterization of extracellular and surface bound adherence proteins of *Staphylococcus aureus* Torgny Schennings

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”The endeavor to understand
is the first and only basis of virtue.”
Benedict Baruch Spinoza

“To Rosemary, Jacob and Linnea with love”

ABSTRACT

Staphylococcus aureus (S.aureus) is a pathogen causing infections. In recent years *S.aureus* formed resistance to most of known antibiotics on the market. It is today a global interest to find alternative treatment against multi drug resistant bacteria.

The course of an infection is a multifactorial process. The first step after invasion is bacterial adhesion. Specific binding proteins are expressed on the cell surface of *S. aureus* with ability to bind different matrix proteins e.g. fibronectin, fibrinogen, collagen, laminin and vitronectin. Immunization with *S.aureus* adhesion proteins, recombinant developed in *E.coli (Escherichia coli)* gave rise to specific antibodies, targeting binding proteins on the cell wall of *S.aureus* and could block bacterial binding *in vitro*. Trials *in vivo,* performed in experimental endocarditis model in rats, further proved a significant protection against infection after immunization.

To survive the environment after adherence and colonization, the invading microorganism need to express proteins interfering with the host immune system. Several extra cellular proteins expressed from *S.aureus* have a plurality of biological effects on the host. Examples of these proteins, investigated in this survey, are Efb (extra cellular fibrinogen binding protein) and Eap (extra cellular adherence protein). Efb bind fibrinogen thus interfering with primary fibrin formation. Efb also bind and inactivate crucial factors in the complement system. These benefits of *S.aureus* are believed to cause delayed wound healing, prolonged bleeding and accessed scar formation found in *S.aureus* infected wounds.

Eap has the ability to bind most of all known matrix proteins. It also serves several functions that interfere with the host immune system. This could be one explanation to the poorly functioning immunologic memory found after an *S. aureus* infection.

The hypothesis in this thesis is mainly based on two theories: Disturbed adhesion primarily affect the virulence of an invading microorganism. Immunization with recombinantly produced adherence proteins stimulate opsonization and make hidden virulence factors visible to the immune system and thus facilitate the ability to clear out infections. The proteins expressed are representative for most common species of *S. aureus*, irrespective of antibiotic resistance.

To mimic the clinical situation in experimental wound infection models it´s important to use small inoculate. It is also important to obtain a strong adaptive immune response with circulating memory cells that consequently would protect against recurrent infections. In the modern way of thinking it is also essential to attack several steps in the course of infection. An experimental wound infection model was thus performed, infected with a minimal infectious inoculate. Multi component immunization was performed with four recombinant proteins, targeted at different steps in the infectious process. The results indicated a significant reduction of bacterial load in the immunized group and a more rapid clearing out ratio compared to the control group after infection challenge with *S.aureus*.

Conclusion: The present studies support the theory that it is conceivable to use immunizations with recombinant extra cellular and cell bound proteins as an alternative to prevent and further on treat infections caused by multi resistant microorganisms.

Key words: *Staphylococcus aureus*, vaccination, surface bound adhesion proteins, extra cellular binding proteins, immunization.

LIST OF PUBLICATIONS

I: T **Schennings T**, Heimdahl A, Coster K and Flock JI Immunization with fibronectin binding protein from Staphylococcus aureus protects against experimental endocarditis in rats. Center for Biotechnology, Karolinska Institute, Novum, 141 57 Huddinge, Sweden and Department of Oral Surgery, Karolinska Institute, Huddinge Hospital, 141 04 Huddinge, Sweden Microbial Pathogenesis Volume 15, Issue 3, September 1993, Pages 227-236.

II: Flock J-I, Heinz SA, Heimdahl A, and **Schennings T**. Reconsideration of the Role of Fibronectin Binding in Endocarditis Caused by Staphylococcus aureus
Department of Microbiology, Immunology, Pathology, and Infectious Diseases[1] and Department of Oral Surgery[2], Karolinska Institute, Huddinge University Hospital, S-141 86 Huddinge, Sweden.
Infection and immunity, May 1996, p. 1876-1878.

III: M. Ryd [b, a], **T. Schennings** [a, b], M. Flock [a], A. Heimdahl [b], and J-I. Flock [a]
[a]Department of Oral Microbiology, Huddinge University Hospital, Huddinge, Sweden[b] Department of Oral Surgery, Karolinska Institute, Huddinge University Hospital, Huddinge, Sweden. Accepted 1 April 1996. ; Streptococcus mutans major adhesion surface protein, P1 (I / II), does not contribute to attachment to valvular vegetation or to the development of endocarditis in a rat model Archives of Oral Biology Volume 41, Issue 10, October 1996, Pages 999-1002.

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V: **Torgny Schennings***[a,b] Filip Farnebo[a], Laszlo Szekely[b] and Jan-Ingmar Flock[b]. [a]Department of Molecular Medicine and Surgery, Section of Reconstructive Plastic Surgery, Karolinska Institutet, Sweden[b], Department of Microbiology, Tumour and Cell biology Karolinska Institutet Stockholm, Sweden. Protective immunization against *Staphylococcus aureus* infection in a novel experimental wound model in mice; APMIS, 2012. **120**(10): p. 786-93.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
CD31	Cluster of differentiation 31
CD54	Cluster of Differentiation 54
Clf-A	Clumping factor A
cnb	Gene encoding collagen-binding protein
Eap	Extra cellular adherence protein
Efb	Extra cellular fibrinogen binding protein
ELISA	Enzyme Linked Immuno Sorbent Assay
Fg	Fibrinogen
*fnb*A	Gene encoding fibronectin-binding protein A
*fnb*B	Gene encoding fibronectin-binding protein B
Fn	Fibronectin
Gal-FnBP	Fused protein D domain FnBP and Galactosidase
GISA	Glycopeptides Intermediately Susceptible Sauer's
GP IIB/IIIA	Glycoprotein IIb/IIIa on the surface of activated platelets
GST	Glutathionetiotransferrase
GST-D	Fusion protein Glutathionetiotransferrase and D domain FnBP
HRP	Hoarse radish peroxidase
ICAM-1	Intercellular Adhesion Molecule-1
IL2	Interleukin 2
LFA-1	Lymphocyte function-associated antigen 1
MAC	Membrane attack complex
mec A	Mobile genetic element *mec*
MHC	Major Histocompatibility Complex
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MRSA	Methicillin-resistant *Staphylococcus aureus*
NBTE	Non Bacterial Thrombotic Endocarditis
NSAID	Non Steroid Anti Inflammatory Drug
OPD	Ortho Phenylenediamine Dihydrochloride
PBP2A	Penicillin binding protein 2A
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween
PCR	Polymerase Chain Reaction

SCC *mec*	Staphylococcal Cassette Chromosome *mec*
StaphVAX	Capsular polysaccharide type 5-and type 8-based vaccine
TCR	T Cell Receptor
TNF	Tumour Necrosis Factor
VISA	Vancomycin Intermediate *Staphylococcus aureus*

"We never broke up, we just took a 14-year vacation"

The Eagles: Hell freezes over 1994

1 INTRODUCTION

1.1 History

Infectious diseases and wound infection have followed humanity during the entire evolution. In 1847 Ignaz Semmelweis described the link between infection, microorganisms and the importance of hygiene [1]. Alexander Fleming (1881-1955) found in 1928 that moulds of Penicillium notatum in contaminated Lurial broth plates *in vitro* stunted growth of *S aureus* [2]. Fleming also demonstrated that injections with pure cultures of this fungus strain inhibited bacterial growth *in vivo*.

Dr. Howard Florey and Dr. Ernest Chain at Oxford University purified the active metabolite of penicillin and small quantities of the preparation could be produced in a powder form. Together with Dr. Andrew J Moyer, Peoria Lab, USA (1941) penicillin could be produced in a larger scale. Clinical trials ended 1943 and the preparation was tested for the first time in wounded allied soldiers after the D-day (invasion of Normandy) June 6 1944. Flemming Florey and Chain was awarded the Nobel Prize *"for the discovery of penicillin and its curative effect in various infectious diseases"* 1945. Dr Andrew J Moyer patented the preparation technique of penicillin in May 1948.

1.2 Antibiotic resistance

Mass production of penicillin was initiated in 1943. Four years later, 1947, the first report was published verifying the presence of antibiotic-resistant *S aureus* strains. Antibiotic resistance created a need to develop new types of antibiotics. Dr. Dorothy Crowfoot Hodgkin mapped the molecular structure of Penicillin and discovered the beta lactam ring (the active structure in beta lactam antibiotics). Different forms of antibiotics could later on be produced e.g. Tetracycline (1955), Nystatin (1957), active against fungal infections and Amoxicillin (1981) [2]. Dr Dorothy Crowfoot Hodgkin mainly conducted the research. In 1964 she was awarded the Nobel Prize in chemistry. Many of these drugs are used widely even today. Penicillin G is still active against infections caused by, e.g. *Streptococci* and *Pneumococci.*

Common characteristic of most penicillin's used today is the limited effect against *S. aureus*. This microorganism has developed resistance, e.g. beta-lactamase (irreversibly

opens the beta-lactam ring) and also resistance against most of the known antibiotics on the market. Antibiotic resistance could be inherited by different *S.aureus* species in but might also be developed, either by spontaneous mutations in chromosomal DNA or by absorption of plasmids (extra chromosomal DNA) from other closely related microorganisms. This feature is also shared with other pathogens e.g. *Haemophilus influenzae*, *Enterococci* and *E. coli* [3].

1.3 MRSA

Antibiotic resistance became an increasingly growing problem in the world [4]. The first penicillinas stabile antibiotic, Methicillin, was introduced 1959. Two years later, in 1961 the first Methicillin-resistant *Staphylococcus aureus* strain (MRSA) were reported from England [5]. Soon thereafter several case reports came from Europe, Japan, Australia, and United States. MRSA is today a global health problem in hospitals around the world and is still an increasing problem. It is believed that the gene *mec*A, encoding the penicillin binding protein 2A (PBP2A), is acquired from distant related species [6].

MRSA is, surprisingly enough, a fairly common species in marine mammals e.g. Bottlenose dolphins [7, 8]. Many authors claim that the resistant strains were contaminants from the environment, but the most likely theory is that the marine mammals from time immemorial are colonized with MRSA. The marine mammals may simply have been used as a natural habitant for this specific microorganism [7, 8].

The gene *mec*A is located in a mobile genetic element called the SCC *mec* (staphylococcal Cassette Chromosome *mec*). The *mec*A gene and the trans membrane β-lactam sensing signal transducer *mec*R1 are regulated by a repressor gene *mec*1. When exposed to beta-lactam antibiotics, *mec*R1 is auto-catalytically cleaved and its metallo-protease domain cleaves in turn *mec*1, which allows the transcription of *mec*A (Fig. 1) [6, 9].

Several different forms have been described, with variation in size and genetic structure [6, 9]. Most MRSA strains are resistant to all known antibiotics, apart from glycopeptides (e.g. Vancomycin).

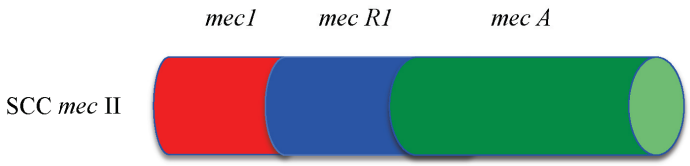

Fig. 1. *Schematic illustration of the SCC mec II, one in of about 20 different combinations of the SSC mec gene.*

MRSA strains also have, during the last decade, shown intermediate sensitivity to Glycopeptides, GISA (Glycopeptides Intermediately Susceptible Sauer's) or VISA (Vancomycin Intermediate *Staphylococcus Aureus*).

VISA, also called "*van*A optimistic *Staphylococcus aureus"*, is expressing the Vancomycin resistance gene *van*A. In the clinic, VISA is defined as a minimum inhibitory Vancomycin concentration of 512 micrograms / ml. The new resistance pattern is now a growing health problem [10]. The relatively few patient cases infected with VISA were all reported to have a known MRSA history, combined with a VRE (Vancomycin Resistant Enterococci) infection or colonization [10]. The Vancomycin resistance gene *van*A from VRE is belived to be taken up by MRSA.

1.4 Ancient proteins

Most genes encoding resistance factors against antibiotics are in fact, like most antibiotics, fairly old [11]. The increasing prevalence of antibiotic resistant bacterial infections seen in the clinical practice is more likely caused by an excessive use of antibiotics in veterinary and human medicine. Analyses of ancient DNA from 30 000 year old Beringian permafrost sediments showed a complete and highly various collection of genes encoding resistance to beta lactam antibiotic, tetracycline and many other antibiotics. Studies on ancient Vancomycin resistance element *van*A confirmed similarity to the structures seen today [11]. The future will likely require new methods to successfully treat and prevent infections caused by multiresistant microorganisms like VISA, and VRE, when conventional therapies are becoming increasingly limited.

1.5 Mechanisms behind infection caused by *S.aureus*

Staphylococcus aureus normally occurs in human skin and in the nasal cavity, without leading to infection. However, infections with *S.aureus,* under different conditions, can lead to a number of serious complications like deep wound infection, sepsis, TSS (toxic shock syndrome) or endocarditis. In surgery, especially Reconstructive Plastic Surgery, secondary infections with *S. aureus* also cause troublesome and long-term complications. The transition from a non-pathogenic colonizing bacterium, to a pathogen, giving rise to infection is a multifactorial process. Several factors are linked to the host. An otherwise immuno compromised patient is at a greater risk of infection. In *S.aureus* a number of more or less well-characterized pathogenicity and virulence factors are present. These include extracellular catalytic enzymes such as proteases, DNAse, hyaluronidase and stafylokinase. In addition also proteins like coagulase (stimulates fibrin formation) and various toxins such as leucocidin (lytic effect on leukocytes), α-toxin and enterotoxins (Fig. 2).

Staphyloxanthin [12] is responsible for the characteristic golden colour that gives *S. aureus* its specific name. Staphyloxanthin also acts as a virulence factor, with an antioxidant action helping the microbe to survive reactive oxygen species produced by the host immune system (Fig. 3).

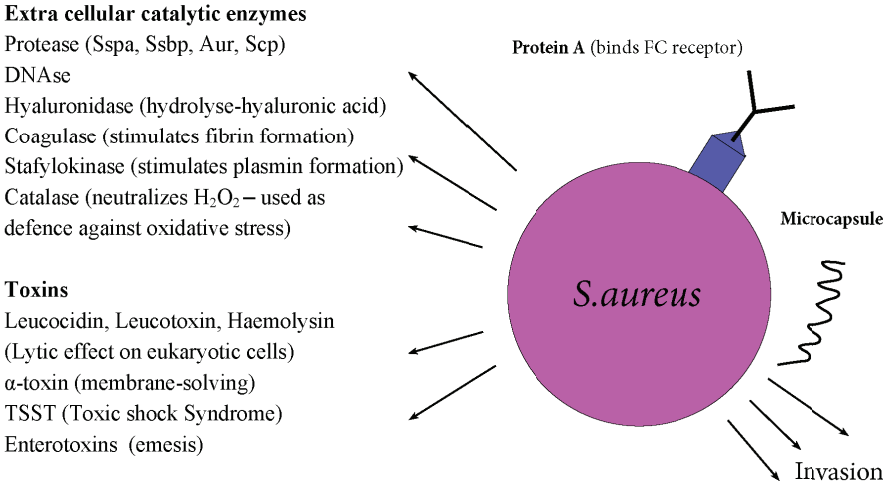

Fig. 2. Virulence factors of S.aureus: Extra cellular catalytic enzymes, Protein A, Microcapsule, Staphyloxanthin and different toxins.

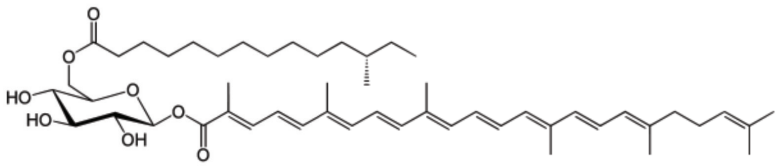

Fig. 3. Staphyloxanthin a membrane-bound carotenoid contributes to the golden colour of S.aureus and provides an increased resistance against oxidative stress [12].

1.6 Specific binding proteins

Another significant factor in the bacterial pathogenesis is ability to bind tissue matrix proteins. Binding of bacteria to different tissues might be the result of an itemized electrostatic or hydrophobic interaction. However, it is shown that most bacterial species studied, express proteins on the surface with specific affinity to different tissue matrix proteins. Surface proteins from S. *aureus* specifically binds to collagen [13], fibronectin [14-18], bone matrix sialoprotein [19], fibrinogen [20-22] laminin [23] and vitronectin [24]. Clf (Clumping factor), binds fibrinogen [21].

1.7 Extra cellular binding proteins

S. aureus also express extracellular binding proteins, with several biological effects on the host organism, e.g. Efb (Extra cellular fibrinogen binding protein) [22, 25], Eap (extracellular adherence protein) [26, 27], ECB (extracellular complement binding protein) [28], SCIN (Staphylococcal Complement Inhibitor) and CHIP (Chemotaxis Inhibitory Protein) [29]. This work has been restricted to studies with Efb and Eap.

1.8 Extracellular fibrinogen binding protein (Efb)

Efb binds to fibrinogen and interfere with the primary fibrin synthesis, resulting in a disturbed coagulation and non-sufficient fibrinogen is formed. Efb also have immunosuppressive properties by binding and disable C3b in the complement system [25, 30]. Blocking C3b in the complement system results in reduced opsonization and also inhibiting formation of the membrane-attack complex (MAC). Forming MAC is a crucial step in the host immune response both in the adaptive immune response (classical pathway), as well as the alternative and Lectin pathway in the innate immune response. The complement binding of Efb appears similar to the complement binding of ECB, despite the ability to bind fibrinogen (see above).

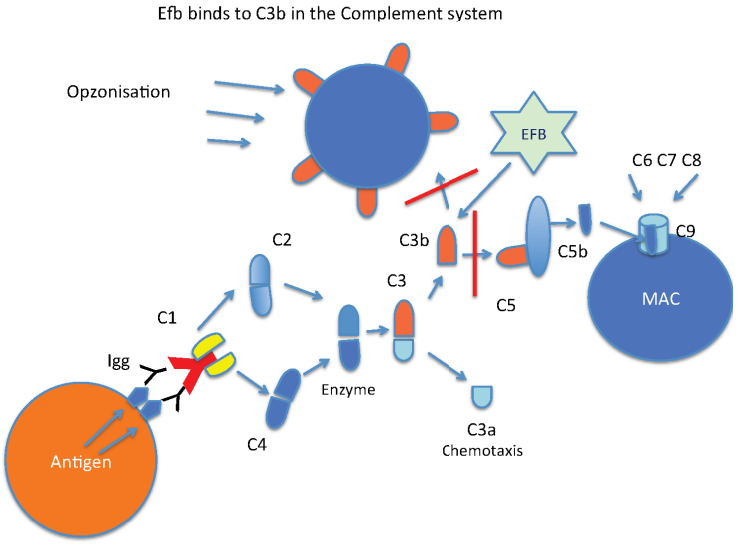

Fig.4. Efb binds and inactivates complement factor C3b, thus preventing opsonization and formation of MAC (membrane attack complex).

Efb also specifically binds to platelets, which prevents aggregation [31]. An experimental stroke model in mice were intravenous injection with epinephrine and collagen injections leading to 100% mortality in untreated mice was used. Mice pre-treated with soluble Efb resulted in total survival [32]. Efb could consequently be an interesting agent in future treatment strategies for prevention of thrombosis in the clinical practice. The interaction between microbes and platelets is claimed to be crucial in endocarditis and other cardiovascular diseases [33].

The extra cellular fibrinogen protein has two binding domains. One is located in the N terminal and one in the C terminal. This function makes Efb unique, with many different binding combinations e.g. Fibrinogen - fibrinogen, Fibrinogen - Glycoprotein GP IIb/IIIa. The binding of Efb to platelets is not directed to Glycoprotein GP IIb/IIIa which is the common way to block platelet aggregation e.g. NSAID, Plavix and monoclonal antibodies (abciximab, Reopro) (Fig. 5). The clinical effect in humans is believed to be a disturbed fibrin formation, with a non-functional fibrin formation. This antiplatelet effect may explain the retardation of wound healing associated with Efb in *S. aureus* wound infections.

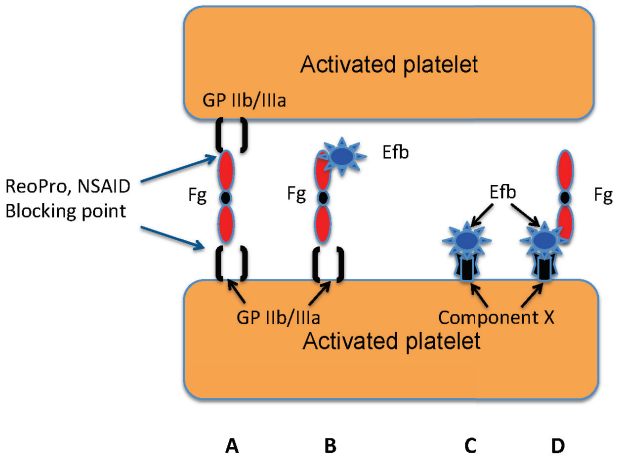

Fig. 5. Binding between activated platelets and Efb. A: The classical fibrinogen binding to glycoprotein GP IIb/IIIa on activated platelets. GP IIb/IIIa is also the target for NSAID, Plavix and monoclonal antibodies (Reo Pro). B: The direct binding of Efb to fibrinogen, which blocks interaction between activated platelets. C: Direct binding of Efb to a still undefined factor on the activated platelet (Component X). D: Binding of Efb to Component X, stimulating additional fibrinogen binding. Fibrinogen binding which is mediated by Efb is "non productive" in the sense that this fibrinogen is unable to bind to GP IIb/IIIa [31].

1.9 Extracellular adherence protein

The mechanisms of Eap are not fully understood, besides its ability to bind a majority of all known matrix proteins. Eap also have several immunosuppressive properties [34, 35]. Eap binds to ICAM-1 (Intercellular Adhesion Molecule-1) also known as CD54 (Cluster of Differentiation 54) on endothelial cells thus interfering with angiogenesis [36, 37]. The ICAM-1 binding also blocks the beta 2-integrin dependent neutrophil recruitment. This binding inhibits leukocyte infiltration in wounds and thereby prolongs the wound healing process [37]. Eap reduces the adhesion of neutrophils to immobilized ICAM-1 and transmigration of TNF (Tumour Necrosis Factor) - enabled human endothelium [38, 39]. Eap is vital in the internalization between eukaryotic and bacterial cells and is thus promoting the direct binding process [27, 40].

A wide range of biological effects regarding interactions between Staphylococcus aureus extra cellular adherence protein (Eap) and host components is reported. Experimental mouse models have previously shown that injections with Eap, providing an inactivation of T cells, results in an inhibition of experimentally produced neuro inflammatory diseases such as MS (multiple sclerosis) [38], RA (rheumatoid arthritis) and Psoriasis [41, 42]. Eap also specifically interact with recombinant full-length OPN (osteopontin also known as bone sialoprotein I (BSP-1 or BNSP)) and the 40 kD N-terminal MMP (matrix metalloproteinase) cleavage fragment and thereby inhibit the migration of cancer and bone metastasis in human breast cancer [43].

1.10 Immunity against *S.aureus*

Adaptive immune response after an *S.aureus* colonization and infection is normally observed in healthy humans [44]. Circulating IgG antibodies can be demonstrated, targeting surface antigens, such as teichoic acid, clumping factors A and B, and bone sialoprotein-binding protein. Also antibodies are formed against extracellular proteins such as alpha-toxin, lipase, enterotoxin A, toxic shock syndrome toxin, scalded-skin syndrome toxin, Eap and Efb. Variations between younger and elderly are found and some individuals are fairly "good responders" to several antigens, while others are "poor responders." [44]. Obviously these antibodies are not protective enough since all humans could get infected with *S.aureus* and yet be recurrently infected with the same pathogen [45].

As the bacteria adhere and multiply, expression of extra cellular proteins, interfering with host defence mechanisms is essential to the microorganism to survive and maintain infection. This might be the cause of long-term colonization, prolonged wound healing, severe scar formations and absence of efficient immunologic memory.

1.11 The role of Eap blocking ICAM-1 / LFA 1 interaction

Week non-specific selectin molecules P, E, and L selectin mediate the first stage of leukocyte-endothelial interaction. Inflammatory response will up regulate the expression of ICAM-1 thereby increasing the adhesive nature of leukocytes and endothelial cells. The selectins initiate a “rolling behaviour” of the leucocytes over the endothelial layer. ICAM-1 then interacts with leukocyte LFA-1 (Lymphocyte function-

associated antigen 1) and stabilizes the leukocyte on to the endothelial layer. The next step is extravasation, the process were the leucocytes are crossing the endothelial layer, also called diapedesis. During diapedesis the PECAM-1 (Platelet / endothelial cell adhesion molecule 1) also named CD31 (cluster of differentiation 31) serves as a "gatekeeper" during the process. CD31 is expressed both on leukocytes and in the intercellular junctions of endothelial cells [46]. It is important to notice that the ICAM-1/ LFA-1 interaction is not specific compared to the specific binding expressed on the bacterial surface (see above) and is only regulated by cytokines and factors concerning the inflammatory response [47].

As the first step in antigen recognition Cytotoxic CD8 T cells use the previously mentioned non-specific interaction between ICAM-1 and LFA-1. This interaction allows the T cell time to align the T cell receptor (TCR) with the MHC class I peptide complex (Major Histocompatibility Complex). A successful TCR interaction will thus increase the adhesive force and initiate the effector function [48]. Conversely, an unsuccessful TCR interaction will not provide adequate adhesive forces and the T cell will simply leave the environment. Analogous to the CD8-target cell / ICAM-1 interaction, also other APC (antigen presenting cells) express ICAM-1 binding, but in this case to the MHC class II molecule. APC also use ICAM-1 to hold CD4 T cells allowing time to initiate activation via interaction of the TCR and the MHC II peptide complex [49]. Eap binds ICAM-1 on activated endothelium and blocks the beta 1 and 2 integrin on to leukocyte. This effect suppresses the cytokines, TNF - alpha (Tumour necrosis factor) and IL2 (Interleukin 2) signalling preventing the trans endothelial migration (see above). Suppression of the cytokines TNF-alpha and IL-2 hence lead to an inactivation of both CD8 and CD4 lymphocytes. This could be one explanation to the weak immunologic memory after an *S.aureus* infection.

1.12 Escaping the immune system

Down regulation of ICAM-1 expression is related to different malignancies e.g. malignant melanoma, lymphoid and myeloid malignancies as well as allergic asthma, atherosclerosis, ischemia, neurological disorders, and organ transplant rejection [49]. As a well-known factor, human cancer has the ability to escape the immune system preferably via down regulation of cytotoxicity and up regulation of supressing factors involving IL-10 (interleukin 10) and TNF-beta (tumour necrosis factor beta). This will

lead to an overexpression of regulatory T_4 helper cells or T-reg (regulatory T- cells). In the environment of a solid cancer oxidative stress factors are expressed. Cytotoxic CD-8 T-cells has proven to be significantly more sensitive to oxidative stress, compared to the regulatory T-cells [50] and consequently creates an immunosuppressing environment. In the surroundings of a chronic abscess caused by *S.aureus,* several proteins are expressed that contribute to increase the oxidative stress in the environment. It is therefore not inconceivable that the immunological response in a bacterial infected environment also is converted into a suppressive state.

It can be hypothesized that neutralizing antibodies directed against adhesive and immuno modulating properties in the microorganism, like FnBP, Clf Eap and Efb, would result in a stronger immune response after immunization. Antibodies would make "hidden" virulence factor visible to the immune system, which would result in a milder infection and an infection easier to clear out via the innate and adaptive immune system. It has also been shown that super-antigenic *S.aureus* stimulates Il 17 (interleukin 17) production from old CD 4 cells but not from young CD 4 cells which proves that memory cells actually are present after an *S.aureu*s infection [51], but they are obviously not protective against relapse [52-54]. Immunizations with recombinant proteins obtain a strong adaptive immune response and might also contribute to persisting memory cells that eventually would protect against recurrent infections.

1.13 Endocarditis

In Swedish material 29% of verified and 60% probable endocarditis are caused by viridans streptococci [55]. In almost all cases, the bacteria originate from the oral cavity [56] and in about half of these cases endocarditis was preceded by some kind of oral surgery [57]. The name viridans streptococci used in most articles is a synonym to Alfa streptococci. The following groups are part of the streptococcal family, associated with sub-acute endocarditis: *Streptococcus sanguis* type II, *Streptococcus mutans*, *Streptococcus mitior* and *Streptococcus milleri*.

In addition to viridans streptococci, endocarditis is also caused by *Staphylococcus aureus*. This microorganism occurs mainly in the nose and on the skin [58, 59]. In the oral cavity, growth of *S. aureus* is present in approximately 20% of healthy "normal people" but in a higher rate in elderly, with other disorders and in young children [60, 61]. Endocarditis caused by *Staphylococcus aureus* from wound injury in the skin is

fairly common on the right heart side without valvular deformities, e.g. in intra venous drug abusers [62, 63]. Bacteraemia occurs after surgery and from heavily infected wounds or infected deep burn injuries. Bacteria can be disseminated out into the blood stream, mainly through the lymphatic system. These microorganisms are usually eliminated by the reticuloendothelial system within a few minutes. In patients with valvular deformity or other organic heart diseases with sterile vegetations, circulating bacteria may adhere and cause endocarditis [64].

1.14 Recent vaccination trials during a decade 1996-2006

It is well known that an infection with *S.aureus* results in poor immunological protection and recurrent infections are common. Effective vaccines against staphylococcal infections do currently not exist. During the last century many attempts have been undertaken to finally prevent and cure infections caused by *S. aureus*. The number of invasive MRSA infections in the USA was estimated to be almost 100 000 in 2005 [65] and the number is still increasing. Approximately 112 million elective hospital operations were performed in 2005 [65]. Patients undergoing surgery could represent a major target for vaccination against *S. aureus*. Today there are at least seven active vaccination attempts at various stages of clinical development (GlaxoSmithKline/Nabi, Pfizer/Inhibitex, Sanofi Pasteur/Syntiron, Novartis, Novadigm, Integrated Biotherapeutics and Vaccine Research International) [66].

Several reviews have described strategies to make the "optimal" vaccine, as well as highlighting the significant challenges of developing an effective *S. aureus* vaccine [67-70]. The enthusiasm has been hampered by two late-stage failures to develop a safe and effective *S. aureus* vaccine. These previous vaccination attempts were mainly based on tree cornerstones.

- Antigens in the two vaccines are expressed by a majority of *S. aureus* isolates.
- The antigens produce a protective immune response in infection animal models [71, 72].
- Antibodies against these antigens used in passive immunization animal studies, were shown to be protective [71, 72].

Nabi was the first company to take the challenge of testing a *S. aureus* vaccine in humans. The vaccine was based on two capsular polysaccharides type 5 and type 8 (StaphVAX) [71]. In 2005, the project closed down due to failure of significance to

reduce *S. aureus* bloodstream infections in ESRD (end stage renal dialysis) patients. In June 2011 another company, Merck and Intercell were recommended by an independent data safety monitoring board to terminate phase II/III development of a subunit vaccine V710, containing a single antigen IsdB, a *S. aureus* cell surface localized iron regulated protein [73].

Obviously the animal models of *S. aureus* infection used to evaluate early-stage vaccines were not predictive in clinical trials. It also appears, that capsular polysaccharides alone or one single protein antigen are not adequate enough to induce a protective immune response in humans. The use of haemodialysis patients with a suboptimal immune status was also frequently mentioned as a contributing factor to poor efficacy of StaphVAX. Consequently a more appropriate patient population is required to evaluate efficacy of a vaccine.

1.15 Future expectations of vaccines against *S.aureus*

In several publications [66, 74-76] future expectations of efficient *S. aureus* vaccine development are described (Fig. 6).

- Multiple antigens (surface proteins, secreted proteins, toxoids and capsular polysaccharides) should be the dominant scientific approach in future vaccines.
- The biological role of the immune response after immunization against *S. aureus* has to be evaluated in appropriate patient populations.
- Immunization needs to develop targeting antibodies that 1: Directly inhibit bacterial viability and/or toxicity. 2: Antibodies that mediate opsonization. 3: Also a need to develop cell-mediated immunity that stimulate recruitment of phagocytes at the site of the infection.
- A whole-cell *S. aureus* vaccine is also described to be an alternative to full safety and efficacy evaluation.

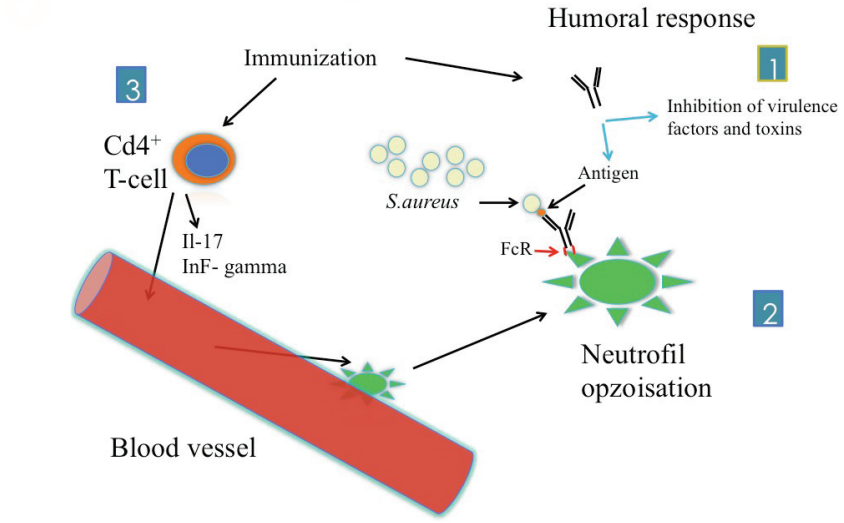

Fig.6. Future expectations of vaccines against S.aureus need to be targeting several different levels in the infectious process. 1. Specific antibodies that inhibit virulence factors. 2. Antibodies that stimulate opsonization 3. Stimulation of the adaptive immune system, to recruit neutrophils into the infected environment.

A significant amount of capital is needed just to invest in clinical approvals. It is fairly unclear if the pharmaceutical companies are capable of investing in an efficacious *S. aureus* vaccine in the nearest future. Concerning the scientific (antigen selection) and clinical approach (patient population) a clinical development program would probably need 15 000–20 000 subjects. At the utmost it is considered to be realistic that an approval might be in order before the end of the decade. But on the other hand the limited success rate in traditional therapies obviously has increased the interest level in vaccine therapies against *S.aureus* infections [66].

"Science is replacing ignorance with confusion"

Nicolaus Copernicus

2 AIMS OF THE STUDY

2.1 The hypothesis of the trials started in the early 1990

Oral surgery usually leads to bacteraemia with a disparate microbiological flora were up to $1x10^2$ different species were detectable using lysis filtration technique [29]. Very few of these species lead to infection or endocarditis and only in predisposed patients with valvular deformity or implanted heart valves. These few species especially, the Viridans streptococci or Staphylococci have characteristics and binding functions, giving them a clear advantage in the infectious process. These pathogens express surface bound proteins able to specifically mediate adherence to extracellular matrix protein [20, 77].

2.2 Specific Aims

1. To increase knowledge about mechanisms in the infectious process and identification of the essential initial mechanisms and functions in the emergence and establishment of infection.
2. To explore the feasibility of using purified surface bound proteins responsible for adhesion to different matrix proteins as vaccination components.
3. To perform usable experimental adhesion models for in *in vivo* adhesion trials.
4. To develop a wound infection model specially adapted for vaccination studies. The model should be simple, standardized and modified with only a small number of bacteria needed as inoculum to establish infection.
5. To evaluate multicomponent immunization with different extra cellular proteins combined with surface bound proteins and to study the biological effects *in vivo* of antibodies targeting different steps in the infectious process.

3 MATERIAL AND METHODS

3.1 Recombinant proteins

The first recombinant protein (Gal-FnBP) used in paper I was previously performed in 1987 [17] as described:

The gene encoding FnBP (fibronectin binding protein) from *Staphylococcus aureus* strain 8325-4 expressed in chromosomal DNA was isolated from a gene bank in pBR322. The original clone containing a 6.5 Kb insert. The region of the *fnb*A-gene encoding the fibronectin-binding activity was identified and sub cloned in an expression vector based on the staphylococcal protein A gene, revealed in two gene products, 87 KD FnBA and 165 kD FnBB. The resulting product in *E. coli* is an extracellular fusion protein consisting of two IgG-binding domains of protein A followed by a fibronectin binding region. The fibronectin binding property is only a 3 x 38 amino acid repeat located in the D-domain. The gene is fused to the gene encoding Galactosidase and ligated into a plasmid inserted into *E. coli.*

3.2 Protein purification

E.coli HB101 with plasmids encoding, either three domains of Eap (henceforth called R13) or Efb-D (extracellular fibrinogen protein fused to the D-domain of the Fibronectin binding protein FnBP) as fusions to His6 tag, was grown in Luria broth at 37 C. IPTG was added at OD600 =1.0 and cultivation was continued until harvest. Lysozyme was added and after centrifugation the supernatant was run through a Ni-conjugated column and eluated with imidazole. The eluate was dialysed over night with PBS. The proteins were run on Phastgel and Western blot analysis and compared with LMW (low molecular weight standard proteins).

3.3 Serum analyses

Serum samples were taken from each animal including the control group after third immunization approximately one week after the last booster. Samples of blood was collected via the tail vein. The antibody titers were analysed by ELISA (Enzyme Linked Immuno Sorbent Assay). Microtiter wells (Costar) were coated over night with appropriate antigen. After rinsing with PBST (phosphate buffered saline with Tween) serum samples were added at serial dilutions and incubated at 1 h 37^{o}. The antibodies were detected with HRP (hoarse radish peroxidase) - conjugated rabbit anti mouse or

rabbit anti rat monoclonal antibodies. OPD (Ortho-Phenylenediamine Dihydrochloride) tablets were used as substrate and stopped with H_2SO_4. The plates were then analysed in an ELISA reader at 492 nm.

3.4 Adhesion trials *in vitro*

Microtiter wells were coated over night with fibronectin, fibrinogen or collagen type II. Various amounts of *S. aureus* were added after pre-treatment with 50µl purified IgG from pooled immune serum or control, non-immune serum. The blocking effect were analysed in ELISA reader [78].

3.5 Experimental endocarditis model in rat.

In this experimental method rats were catheterized with a venflon catheter via a. carotis communis on the right side. The catheter was then introduced on to the aortic valve and ligated for 24 hours. This resulted in a disturbed circulation in the environment surrounding the aortic valve, which resulted in deposition of fibrin thrombin complex directly on to the valve.

The damaged heart valve is primary covered with fibrinogen, while also deeper layers of fibronectin in the tissue are superficially exposed. As no bacteria are present, these structures are named “sterile vegetation” NBTE (Non bacterial thrombotic endocarditis) (Fig.7). This is a condition that also occurs in the clinic, most often in patients having congenital valvular defects or malignant diseases [79-81]. Sterile vegetations are targets for circulating microorganisms with the ability to bind and adhere to fibrinogen and fibronectin in the vegetations. In this experimental endocarditis model the animals were intravenously infected with *S. aureus* via the tail vein. Circulating bacteria can thus colonize the valvular tissue and cause endocarditis. Bacterial adhesion can probably also be mediated by circulating fibrinogen, which binds to both the bacterium and the tissue. Pilot trials were also performed using this experimental endocarditis model were large inoculum 10^7 CFU (colony forming units) of *S.aureus* or *S.mutans* were compared with two non-pathogenic microorganisms, *Bacillus subtilis* and *E coli*, both lacking the ability to bind fibronectin and fibrinogen. The animals were analysed after 1 hour only to study the primary adhesion. This study showed that *S. aureus* and *S. mutans* adhered equally well to vegetation on the damaged aortic heart valve. However, these pathogens differed significantly in their

ability to adhere to the intact right side pulmonary valve. The two non-pathogenic strains could not be detected, neither in vegetation on the left side, nor to the right valve after an hour. This study shows that adhesion to the damaged endothelial surface and vegetation is specific and is available only in some endocarditis pathogenic microorganisms e.g. *S.aureus* and *S.mutans*. The study also shows that *S. aureus* has a superior ability to bind intact endothelial cells compared to the *S. mutans* strain (data not publ.). These data are supported by previous studies confirming the direct binding between *S.aureus* and endothelial cells [78, 82-84].

Fig. 7. Electron microscopy showing aortic valve and a sterile vegetation after withdrawal of the polyethen catheter. Photo: Torgny Schennings.

3.6 Isogenic strains

Isogenic strains were used in paper II, III and IV. In paper II a wild type strain 8325-4 was compared with a double mutant strain DU 5883 [85]. The mutant strain were modified with gene substitutions for both *fnb*A and *fnb*B, encoding two fibronectin binding proteins. A DNA fragment encoding tetracycline resistance was inserted into *fnb*A and a fragment encoding erythromycin resistance was inserted into *fnb*B. The two strains had identical characteristics, apart from the ability to bind fibronectin, Paper II.

In Paper III, a wild type strain (NG8) of *streptococcus mutans* was compared with a mutant strain (832) where the gene encoding Tetracyklin resistance were inserted into the *spa* P gene encoding the surface binding protein PI (I/II) [86].

In Paper IV, clinical isolates Phillips and a mutant strain P100 lacking collagen binding by gene insertion of Gentamycin resistance [87] was used (Fig.8). Collagen adhesin-negative mutant PH100 was constructed by replacing the chromosomal collagen adhesin gene (*cna*) in a clinical strain, Phillips, with an inactivated copy of the gene.

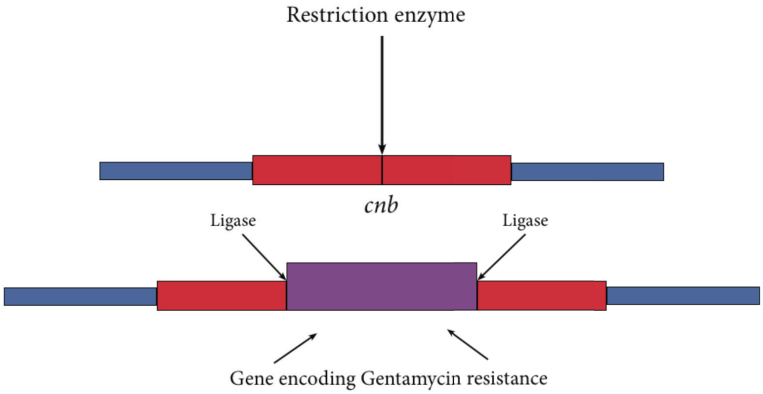

Fig. 8. The gene encoding collagen-binding protein (cnb) is cleaved with restriction enzyme and the gene encoding gentamycin resistance is inserted and ligated. Collagen binding is deleted and replaced with Gentamycin resistance.

3.7 Fusion proteins

To make the protein immunogenic the molecular weight must be appropriate. The first protein used in Paper I the gene encoding FnBP was fused to beta-galactosidase. Immunization with this protein gave rise to antibodies targeting both the D domain and to beta-galactosidase (Fig.9).

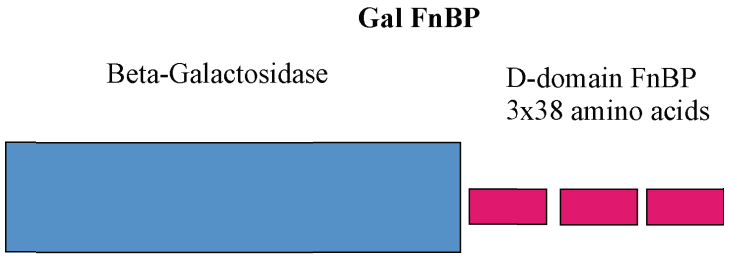

Fig. 9. The gene-encoding Beta - galactosidase is fused to the D domain of the gene encoding fibronectin-binding protein (FnBP). The gene product, Gal-FnBP is then composed of beta-galactosidase, a large molecule 120 kDa, and the D-domains of FnBP, 12,5 kDa.

In paper V, a fusion protein Efb-D (Efb and the D-domain of FnBP) was used. This fusion protein contains two biological active peptides (Fig.10). Immunization with this fusion protein resulted in antibodies with similar biological activity, which means that no antigen dominated over the other. Fusion proteins are also simpler to handle and to purify. Fusion proteins with up to 7 alleles have previously been used [88].

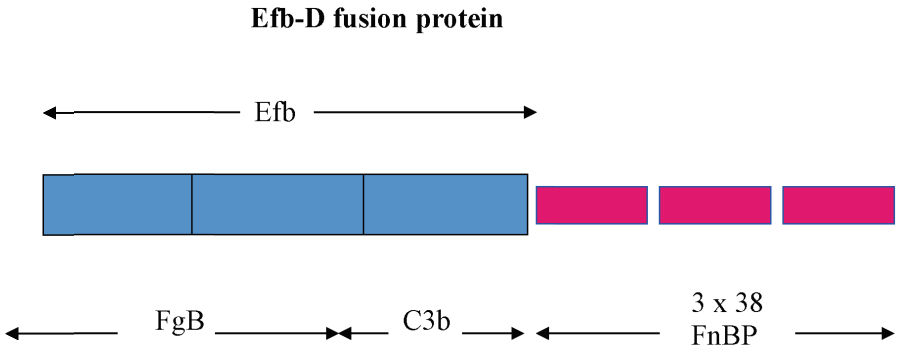

Fig.10. Fused protein Efb and the D-domain of FnBP. Molecular weight 19 kDa Efb and 12,5 kDa FnBP. The immune response was found to be equal after immunization and no protein dominated over the other.

3.8 Wound infection in mice

Consequently a new infectious model in mice was performed. The model is simple sensitive and possible to reproduce. 4.5 mm full thickness wounds were punched out in the gluteal region in female Balb C mice, 22 g. To make the model sensitive, snake venom from *Bothrops asper* was used. 25 µg toxins were injected directly under the muscular fascia combined with a low inoculum 0.5-1 x 10^2 CFU *S.aureus* on the right side wound. The left side were only injected with toxin serving as control and also as a target for haematogenous spread infection (Fig. 11).

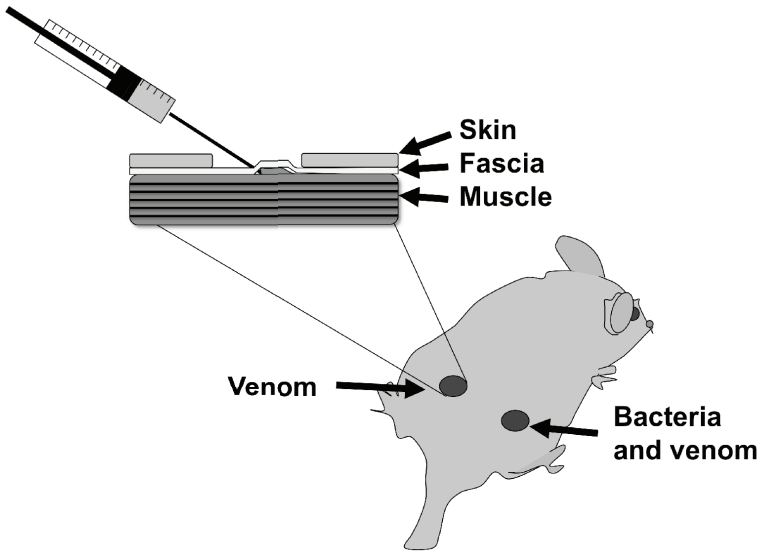

Fig.11. Infection model in mice.

4 RESULTS AND DISCUSSION

4.1 Immunization with fibronectin binding protein from *Staphylococcus aureus* protects against experimental endocarditis in rats (Paper I)

4.1.1 Adhesion trials

To study adhesion *in vitro* and *in vivo* we wanted to clarify the mechanisms behind bacterial adhesion. As the first step in the infection process the bacteria bind to various structures in the host organism, colonize and finally develop infection. On the cell surface of *S. aureus* adhesion proteins are expressed with specific affinity for matrix proteins such as fibronectin, fibrinogen, collagen, vitronectin and laminin. Binding fibronectin has been described to be the major virulence factor of *S. aureus* [64, 89]. The gene encoding the fibronectin binding protein was earlier cloned [17]. The fibronectin binding domain only 3 x 38 amino acid repeats called D-domain. The 3xD encoding DNA was fused with the gene encoding Galactosidase. The fused gene was inserted into a plasmid in *E.coli*, which allowed production in a large scale. The protein was purified and injected into Whistar rats together with Freud's complete adjuvants. The fibronectin binding protein might be too small to be immunogenic by itself but when fused to a larger molecule (Galactosidase) [90] the whole molecule is immunogenic (see materials an methods). Antibodies raised after immunization with Gal FnBp could be obtained in the serum targeting Galactosidase as well as the fibronectin-binding domain.

Immune serum was then used for different trials *in vitro* concerning bacterial adherence to immobilized fibronectin. The immune sera with antibodies directed against the fibronectin binding protein on *S.aureus* whole bacterial cells proved to be able to block out the bacterial binding on to immobilized fibronectin *in vitro* (Fig.12).

The trials showed that the immune response was committed via the adaptive immune system. A booster doze given after 12 months gave a quick response with high IgG antibody titers, indicating B-cell mediated immunological memory that sustained up to 24 months. (Data not shown).

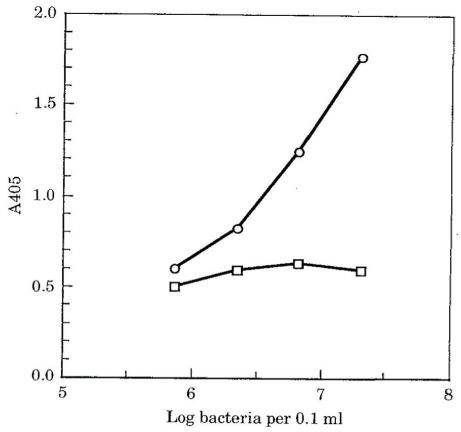

Fig.12. Adherence blocking activity of IgG from Gal-FnBP immunization. Various amounts of S.aureus were added to immobilized fibronectin in Microtiter wells after pre-treatment with 50ul purified IgG from pooled immune sera (boxes) or pooled pre immunization sera (circles). Bacterial adherence was measured as described in Materials and methods.

4.1.2 Experimental endocarditis model in rat

The results we found suggested that a blocking effect *in vitro* is obvious and very promising. The importance of the different adhesion factors involved in the adhesion process resulted in an urging need for an experimental adhesion model *in vivo*. We wanted to achieve a suitable experimental adhesion model. The adhesion of *S. aureus in vivo* was thus studied in an endocarditic model in rats, which in an orderly manner represents pure adhesion [77, 91]. In the experimental endocarditis model a polyethen catheter were induced via the right common carotid artery on to the aortic valve. This resulted in a disturbed blood flow. Sterile vegetations were formed on the aortic valve after 24 hours (see materials and methods). Sterile vegetations contain large amounts of fibrinogen and fibronectin. The experimental endocarditis model was used *in vivo* in immunized and unimmunized control animals. The catheterized animals were infected after 24 hours intravenously via the tail vein with 1 x 10^5 CFU of *S. aureus*. The vegetations found were then homogenized and plated on to Blood agar plates with the lowest level of detection set at 2 CFU [64]. A significant difference in of bacteria adhered after 36 hours infection from immunized and unimmunized animals was found ($p < 0.05$) (Fig. 13) [92].

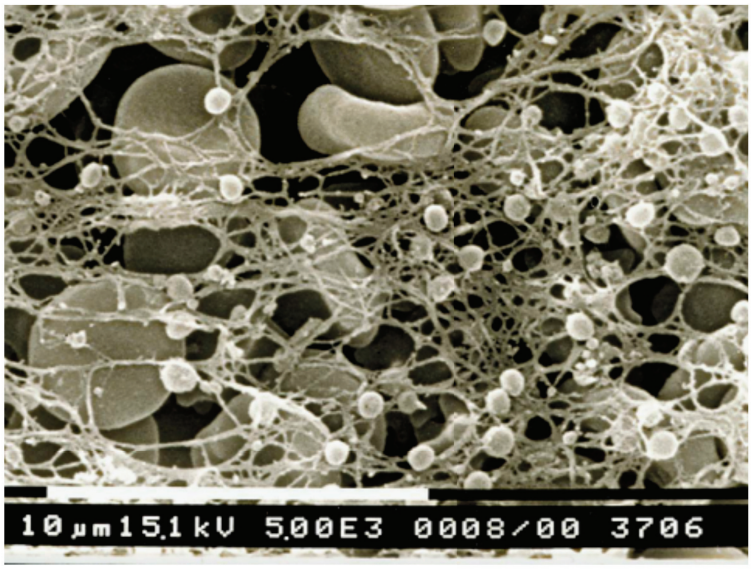

Fig. 13. Electron microscopic image of an infected valvular vegetation showing red blood cells, fibrin formation and adhered S.aureus. Photo T. Schennings.

Serum from immunized animals was pooled. IgG were then purified in protein G - sepharos column. Antibodies from pooled serum could block the adhesion of *S. aureus* whole cell to immobilize fibronectin *in vitro* (Fig. 6). Passive immunization trials *in vivo* were also performed in experimental endocarditis model [91]. In these trials anti-Gal FnBP serum were given intravenously prior to infection, the control group received serum from non-immunized rats. No difference between these groups was shown. Results published in Paper I.

In conclusion the blocking effect after immunization was promising, but in a biological system the conditions is much more complicated and other factors are far more tangible. We found that the number of bacteria adhered were reduced but the microorganisms that finally adhered, could later develop infection (Fig. 14) [92].

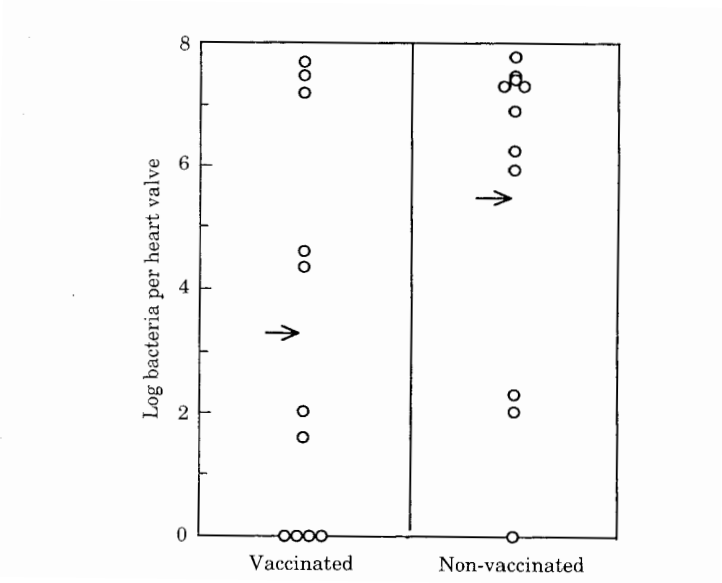

Fig.14. Number of bacteria on left hart valves with attached vegetations in vaccinated or non-vaccinated animals. Arrows indicate the mean log values.

4.2 Reconsideration of the Role of Fibronectin Binding in Endocarditis Caused by Staphylococcus aureus (Paper II)

The previous study showed that immunization with the fibronectin binding protein could protect against experimental endocarditis. The consensus during this time period was obvious that the fibronectin binding availability of *S. aureus* was the key to primary adhesion [64, 89]. Even today it is thought that fibronectin binding in the initial phase of an infection is of greatest importance [93]. To prove this theory we performed a similar study without immunizing. Two isogenic strains were used. Wild type 8325-4 and DU 5883, a double mutant strain with gene deletions in both of the fibronectin binding domains (see materials and methods). The gene substitution resulted in a double mutant strain FnBP A- FnBP B-. A DNA fragment encoding tetracycline resistance was inserted into *fnb*A and a fragment encoding erythromycin resistance was inserted into *fnb*B [85]. Adhesion trials were performed in *vitro* where the two isogenic

strains were compared. The mutant strain was unable to adhere immobilized fibronectin but both strains adhered equally on to fibrinogen.

Attempts were also carried out *in vivo* in a rat model using a catheter-induced infectious endocarditis. No differences were detected between the two tribes after an hour or after 24 hours irrespectively of inoculum doze. The results strongly suggest that bacterial adherence to damaged heart valves is of a multifactorial nature, and other binding functions are likely to compensate for the lack of fibronectin binding in *S. aureus* (Fig.15). Paper II [94].

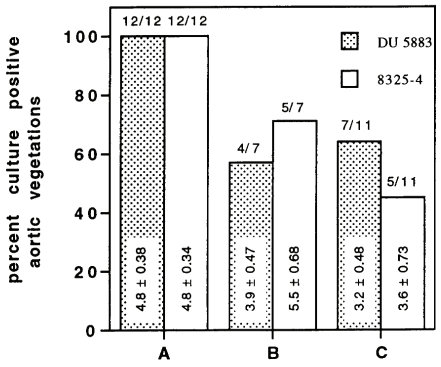

Fig.15. Rate of infection as function of the inoculum size. The percentage of animals with endocarditis, defined as culture-positive vegetations, was plotted for each inoculum size: 10^6 (A), 10^5 (B), and 10^4 CFU (C). The numbers above the bars indicate the number of infected animals per the total number of animals in a specific group. The numbers within the bars are mean log CFU and standard errors of culture-positive vegetations.

The positive results found in Paper I, besides blocking, could rather be explained by the effect after immunization and the obvious opsonizing effect, which further on was proved in other studies, presented later [95].

4.3 *Streptococcus mutans* major adhesion surface protein, P1 (I / II), does not contribute to attachment to valvular vegetation or to the development of endocarditis in a rat model (Paper III)

In human endocarditis, the most common pathogen is the viridans streptococci, causing approximately 50 % of all the clinical cases [55, 96]. Oral viridians streptococci e.g. *Streptococcus mitis* and *Streptococcus sanguis* deemed to have caused 30-50% and *Streptococcus aerginosus* and *Streptococcus mutans* 8-18% of all viridans endocarditis cases. The Viridans Streptococci are mainly colonizing the oral cavity. After different types of oral surgery these pathogens can disseminate into the blood circulation and further on bind to sterile vegetations on the heart valvular tissue [97]**.** This is proposed to be the cause of the overrepresentation of Viridans streptococci in human endocarditis. *S. mutans***,** usually associated with dental infections, e.g. dental caries, expresses a 185 kDa surface bound adhesion protein known as antigen P1 (I / II) [98]. This protein binds specifically to the enamel and dentin on the tooth surface. There may be a similarity between PI antigen that binds to the tooth surface with the 97 kDa sialo binding protein expressed in *S. aureus* [99]. The sialo binding protein indeed binds to dentin on the tooth surface too, but preferably to bone matrix structures causing osteomyelitis and septic arthritis. Sialo binding protein is a non-collagen binding protein and binds to crystalline structures in bone matrix, like uncovered dentin and sclerotic cartilage. In the clinical situation the tooth surface, a salivary pellicle consisting of a plurality of salivary and plasma proteins and also large amounts of fibrinogen and fibronectin cover both enamel and dentine. The pellicle is the main targets of the binding protein PI in *Streptococcus mutans*.

The PI antigen is immunogenic, and low levels of antibodies are found in normal sera in man [100]. Increased antibody titer IgA and IgG to antigen P1 are found in patients with *S. mutans* endocarditis [101]**.**

Attempts has also been made to immunize against dental caries with PI/II, actually with limited success [102]. Whether this protein is causing adhesion of *S.mutans* on to injured heart valves was previously never investigated. The gene encoding the PI antigen has been cloned and sequenced [103]. A mutant of *S. mutans* (834) is lacking the gene (*spa*P) encoding the P1-surface antigen. The gene encoding Tetracyklin resistance were inserted into the *spa* P [104]. The fibronectin, fibrinogen and the

collagen binding capacity in the mutant strain (832) were compared with the isogenic wild type strain (NG8) *in vitro.* Both strains were unable to bind fibrinogen but they bound equally to collagen type II. Surprisingly the mutant strain bound twice as well to immobilized fibronectin compared to the wild type strain. This phenomenon was probably an effect of the gene insertion, which probably resulted in an overexpression of the fibronectin-binding domain. The binding was still weak in both strains and is most likely lacking clinical significance. Both strains were also compared and found to be equal in clearing out ratio after 1-hour intravenous infection.

In the experimental rat endocarditis model the number of bacteria cultured from vegetations after 1 h adhesion trial and 48 h with manifest endocarditis were compared between the two strains. The results after growing vegetations clearly showed that no difference could be detected between the two strains. It appears that Pl antigen is irrelevant regarding primary adhesion and also as a virulence factor in endocarditis caused by *S. mutans* (Fig.16) [86].

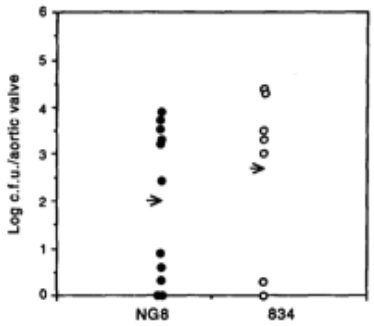

Fig.16. Log number of bacteria recovered from aortic heart valve vegetations after infection with S. mutans strains NG8 and 834. Arrows indicate mean values. Symbols: closed circles strain NG8, open circles strain 834.

4.4 Collagen binding of *Staphylococcus aureus* is a virulence factor in experimental endocarditis (Paper IV)

Based on the findings in paper II we concluded that the importance of the fibronectin binding capacity was a bit overestimated and many other factors were probably involved in the primary binding process. Further analyses of valvular tissues were performed. Vegetations caused by mechanical heart valve damage, intact valvular tissue from the right side and aortic endothelium were removed from catheterized animals, and non-catheterized animals respectively. The tissues were homogenized and analysed with Western-blot technique with conjugated specific antibodies. Collagens type II proved to exist in sterile vegetations (data not shown). Similar finding have also been described in earlier publications [105].

In the present study two isogenic strains of *S. aureus* were used. A clinical strain, Phillips and a mutant strain P100 lacking the collagen binding capacity by a gene insertion of Gentamycin resistance [87]. Collagen adhesin-negative mutant PH100 was constructed by replacing the chromosomal collagen adhesin gene (*cna*) in a clinical strain, Phillips, with an inactivated copy of the gene (see materials and methods).

The results after the *in vitro* adhesion studies between the two strains showed that the difference in collagen binding were obvious. The binding capacity on to stationary fibronectin and fibrionogen did not differ between the two strains.

The two isogenic strains Phillips and PH100 were further on compared *in vitro* experiments in a rat model for catheter-induced infectious endocarditis. Separate groups of rats with traumatized aortic valve were infected intravenously with either of the two strains. After 1 hour of adhesion, we found no difference between the two strains (fig. 17). In rats sacrificed 24h after challenge the collagen binding strain was significantly more virulent compared to the mutant strain ($p < 0.001$). The results were confirmed by further infection with a 1:1 mixture of the parent strain and the mutant as inoculum. In a mixed infection study the non-collagen binding strain were equal in adhesion after one-hour exposition. In 24h and 48h trials the wild type of *S.aureus* was superior to the mutant. This findings supported the theory that collagen binding is of limited importance concerning primary adhesion but of a grater importance in the next step, in other words in the establishment of infection (Fig.18 and 19) [106].

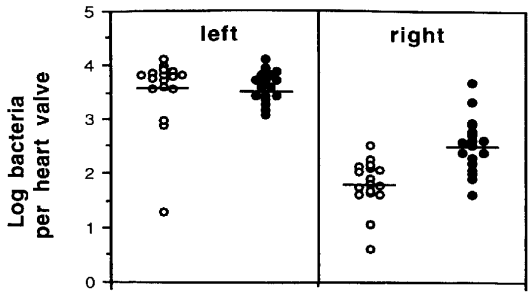

Fig.17. No of bacteria recovered from aortic (left) and pulmonary (right heart valves after 1 hour. Horizontal lines indicate mean values. S.aureus Phillips (filled circles) and S.aureus PH 100 (open circle).

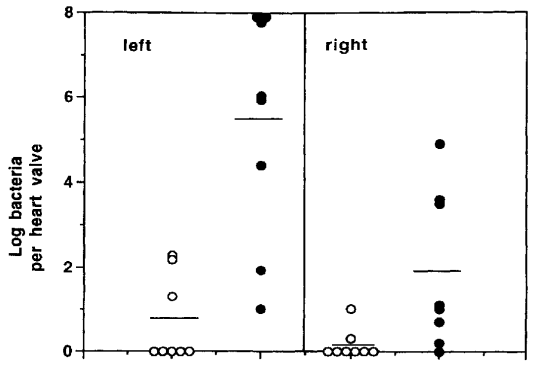

Fig.18. No of bacteria recovered from aortic (left) and Pulmonary (right) valves after 24 hours. Horizontal lines indicate mean values. Bottom line indicate < 2 CFU recovered from each valve (detection limit). S.aureus Phillips (•) filled circle, S.aureus PH 100 (O) open circle.

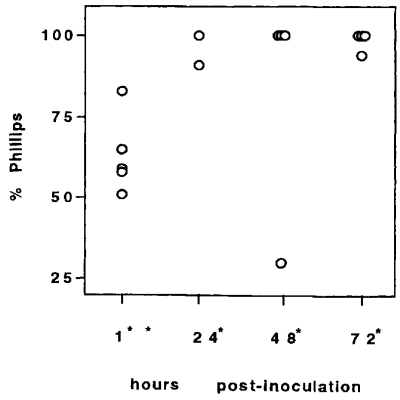

*Fig.19. Percentage of S.aureus strain Phillips recovered from aortic valves with vegetations at various times after animals were infected with a mix 1:1 of Phillips and PH 100. The total amount of recovered bacteria from each valve was estimated and compared with ratio in inoculate (X^2 test). P < * 0,01 and ** 0,10 vs. inoculum.*

To prove our theory the *cna* gene was also reintroduced into the collagen binding protein deficient mutant PH 100 on an autonomously replicating plasmid using a polymerase chain reaction amplified DNA fragment encompassing the *cna* gene. The resulting strain, called PHC, was tested *in vitro* in adherence trials on to stationary collagen, fibronectin and fibrinogen proving that collagen binding capacity was regained intermediate to that of Phillips and PH100. PHC was also used *in vivo* in a mixed-inoculation experiment (PH100 and PHC). The plasmids were lost at a high rate, probably because no antibiotic selection pressure could be applied *in vivo*, and therefore the results were inconclusive (data not shown).

4.5 Protective immunization against *Staphylococcus aureus* infection in a novel experimental wound model in mice (Paper V)

Previous studies were mainly focused on primary bacterial adhesion blocking. The process in the establishment of an infection is obviously a bit more complicated. We wanted to create an infection model that could mimic the clinical situation as closely as possible, where damaged and avascular tissue is infected with a very small inoculum. The objective of further immune studies was to create a strong antibody response directed against mechanisms of *S. aureus* that interfere with the host's defence mechanisms. We particularly desired to produce an immunization method completely independent of resistance to antibiotics in the species. In addition to the previously mentioned adhesion proteins, S. *aureus* express extracellular proteins with ability to act and interfere in different stages during the infection process and also interfere with the host defence mechanisms e.g. Eap (extracellular adherence protein) and Efb, (extracellular fibrinogen binding protein). Efb has the ability to interfere, as well with the fibrin syntheses, as with complement activation (by binding to factor C3b).

To assess the effect of multi-component immunization against Staphylococcus aureus infections a new experimental wound infection model in mice was developed. Before infection the animals were immunized with four recombinant *S. aureus* proteins expressed in *Escherichia coli*. 1: R 13 (domain 1-3 of extracellular adherence protein EAP), 2: Efb - D (a fusion protein containing extracellular fibrinogen binding protein (EFB) and the fibronectin binding D-domain of the fibronectin binding protein (FnBP) and 3: Clumping factor A (Clf A) which previously were proved to be important in the infectious process by binding fibrinogen [107].

Necrotic lesions were induced in mice by injecting venom from *Bothrops Asper* (Nicaragua) containing lysine-49 phospholipase A2 [108]. The toxin causes necrosis in humans and in the clinical situation the wounds habitually get secondarily infected with *S.aureus*. Further on the animals were infected with a low inoculum *S.aureus* strain Phillips ($1x10^2$ CFU). The wounds were swabbed and cultured day 3 and day 5, when the wounds were excised and analysed histologically.

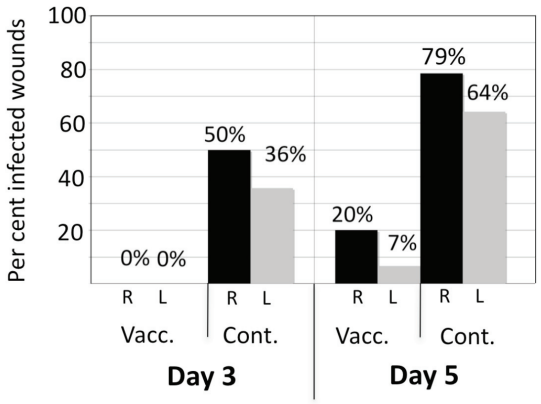

Fig. 20. Bacteria recovered from infected wounds after 3 and 5 days after challenge. After 3 days undisturbed crust formation was observed in all immunized animals. After 5 days the wounds were cultured and histologically examined showing a faster clearing out ratio in the vaccinated group compared to the control group (P< 0,001).

In the immunized group, we found a significantly lower bacterial colonization, uninterrupted crust formation and a quicker healing process compared to the unimmunized control group (Fig.20) [109].

According to contemporary authors a vaccine today needs to target different steps in the infectous process (Fig.6). The first property of a vaccine, is to interfere with virulence factors, like adhesion blocking e.g. FnBP (Paper I, V) and Clf (Paper V).

A second characteristic is capability to stimulate opsonization. Imunizations in Paper I and Paper V, obviously stimulated opsonization. In addition, the properties inhibiting direct opsonization found in e.g. Efb can also be neutralized with antibodies.

The third nature of a vaccine is upregulation the adaptive immune system of $CD4^+$ T-cells and release of IL 17 and InF-gamma to stimulate neutrofil infiltration into the infected environment. Immunization in Paper I, indicated circulating memory cells and

a probable adaptive immune response. It is still unclear if the third mission of a vaccine is accomplished in this work. On the other hand, the blocking effect on diapedesis seen in Eap – ICAM 1 binding is presumably neutralized with antibodies directed to Eap (Paper V). This would in any case not prevent the recruitment of neutrofil cells into the infected environment. The combination of multicomponent vaccination used in Paper V might be one key to find alterative tratment strategies against multi drug resistant *S.aureus* infections.

"I do not like antibiotics. They kill bacteria!"

Roland Möllby

5 CONCLUSIONS

- The hypothesis in this thesis is based on two theories:
  1. Disturbed adhesion primarily affect the virulence of an invading microorganism.
  2. Immunization stimulate opsonization and make hidden virulence, and immunomodulation factors visible to the immune system, and thus facilitate the ability to clear out infections.
- Immunization with *S. aureus* adhesion proteins gave rise to specific antibodies, targeting binding proteins on the cell surface. A blocking effect *in vitro* was demonstrated. In experimental endocarditis model *in vivo,* a significant protection against infection after immunization was showed (Paper I).
- A gene deletion in the fibronectin binding protein disabled *S. aureus* to bind immobilized fibronectin. Lacking fibronectin binding did not reduce virulence in experimental endocarditis *in vivo* (Paper II).
- Endocarditis pathogen *Streptococcus mutans* lacking the PI (I/II) did not reduce bacterial binding or virulence in experimental endocarditis *in vivo* (Paper III).
- *S.aureus* lacking the ability to bind collagen did not affect primary adhesion in experimental endocarditis model *in vivo*. However, it proved to be of considerable importance during the maintenance of an infection. (Paper IV).
- Blocking antibodies against a single surface bound binding protein could eventually reduce the infectious load but bacteria adhered could as well later on establish infection. The significant reduced infection after immunization seen in paper I is probably not a blocking effect but rather a result of opsonization.
- The experimental endocarditis model is a pure adhesion model. To study the infectious process more deeply, a sensitive wound model is more suitable to mimic the clinical situation.
- Multicomponent immunization used in Paper V, gave rise to antibodies targeting different steps in the infectious process. A significant lower bacterial load and faster clearing out ratio was found in immunized group. The antigens used in Paper V could be one step in the right direction to find a working combination of effective vaccine components against infections caused by *S. aureus.*

6 FUTURE PERSPECTIVES

Henceforth, we can expect that fused adhesion proteins could be used as immunization components against staphylococcal infections, passive or active immunization can be used as protective agents against establishment of infection and endocarditis in certain risk groups irrespectively of antibiotic resistance. To make a suitable vaccine against *S.aureus* it is important to find appropriate antigens, representative of most common pathogens. In Paper V all four antigens are expressed from the majority of *S.aureus*. All antigens recombinant produced are originally expressed and preserved in the chromosomal DNA in *S.aureus,* irrespective of antibiotic resistance.

It is also interesting to find specific pathogens in different infections. What differs the pathogen in a burn injury after three days in hospital, from to the strain in the patients nose the moment before the accident? Knowledge of the specific pattern of a pathogen could ultimately lead to tailored immunization before e.g. an upcoming surgery or organ transplantation.

Vaccination with recombinant proteins, alternatively, passive immunization with monoclonal antibody could inhibit the virulence and facilitating the elimination of invading microorganisms. The infectious model in mice used in Paper V was made sensitive with *Bothrops asper* snake venom and the infectious dose could be minimized to 0.5-1.0 x 10^2 bacteria. It is probably important to as closely as possible mimic the clinical situation. In future trials we are planning to use a diabetes model in mice with a knock out gene encoding the leptin receptor. In this diabetes model spontaneous infections occur with Gram-positive bacteria. This model could represent an interesting challenge in vaccination trials.

The use of Efb in clinical trials specifically directed against anticoagulation would be an exciting challenge and could be used to protect clotting and ischemic conditions. In Reconstructive Plastic Surgery, Efb could be used to extend the ischemic time in e.g. a free graft.

Eap is also an interesting protein with a variety of immunosuppressing properties. Eap binding to ICAM-1cold be used in the clinical situation. Today there are two approaches to ICAM-1 therapy:

1. Antibody-mediated neutralization of ICAM-1 or LFA-1 with an inhibition of cytotoxic CD8 T-cells in autoimmune conditions.

2. Pharmacological induction of ICAM-1 with recruiting cytotoxic CD8 T-cells in e.g. malignant conditions and infections.

One of these opposite treatments is administered depending on the type of disease. Studies using Eap to utilise the ICAM-1 blocking effect proves a similarity with monoclonal antibodies targeting ICAM-1[38] and could be used in the treatment of multiple sclerosis (MS), allergic asthma and also Rhinovirus infections. Also Eap binding the nuclear factor (NFκB) [37] with an inhibition of tube formation thus preventing angiogenesis is also an interesting perspective in the future as treatment of solid cancer and distant metastasis.

S.aureus interference with the immune system might henceforth prove to influence other conditions, from chronic wounds to malignancies. It is interesting to understand why the extracellular protein Efb blocks out important steps in the complement system by its binding and inactivation of C3b. The MAC complex does not eradicate Gram-positive capsular forming microorganisms like *S.aureus*. On the contrary, it is not unlikely that *S.aureus* paves the way for other pathogens to colonize wounds in different conditions e.g. diabetic foot ulcers. Further studies in this area are likely to open new opportunities for exciting research.

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